Plant Breeding **124**, 525—531 (2005)

Molecular mapping determines that Hessian fly resistance gene *H9* is located on chromosome 1A of wheat

L. Kong[1], H. W. Ohm[1,3], S. E. Cambron[2] and C. E. Williams[2]

[1]Department of Agronomy, 915 W. State St., Purdue University, West Lafayette, IN 47907; [2]Crop Production and Pest Control Research Unit USDA-ARS-MWA and Department of Entomology, Purdue University, West Lafayette, IN 47907, USA; [3]Corresponding author, E-mail: hohm@purdue.edu

With 5 figures and 2 tables

Received January 28, 2005/Accepted March 30, 2005
Communicated by R. A. McIntosh

Abstract

Hessian fly [*Mayetiola destructor* (Say)] is one of the major insect pests of wheat (*Triticum aestivum* L.) worldwide. Hessian fly resistance gene *H9* was previously reported to condition resistance to Hessian fly biotype L that is prevalent in many wheat-growing areas of eastern USA and an RAPD marker, $OPO05_{1000}$, linked to *H9* in wheat was developed using wheat near-isogenic lines (NILs). However, marker-assisted selection (MAS) with RAPD markers is not always feasible. One of the objectives in this study was to convert an RAPD marker linked to the gene *H9* into a sequence characterized amplified region (SCAR) marker to facilitate MAS and to map *H9* in the wheat genome. The RAPD fragment from $OPO05_{1000}$ was cloned, sequenced, and converted into a SCAR marker $SOPO05_{909}$, whose linkage relationship with *H9* was subsequently confirmed in two F_2 populations segregating for *H9*. Linkage analysis identified one sequence tagged site (STS) marker, *STS-Pm3*, and the eight microsatellite markers *Xbarc263*, *Xcfa2153*, *Xpsp2999*, *Xgwm136*, *Xgdm33*, *Xcnl76*, *Xcnl117* and *Xwmc24* near the *H9* locus on the distal region of the short arm of chromosome 1A, contrary to the previously reported location of *H9* on chromosome 5A. Locus *Xbarc263* was 1.2 cM distal to *H9*, which itself was 1.7 cM proximal to loci *Xcfa2153*, *Xpsp2999* and *Xgwm136*. The loci *Xgwm136*, *Xcfa2153* and $SOPO05_{909}$ were shown to be specific to *H9* and not diagnostic to several other Hessian fly resistance genes, and therefore should be useful for pyramiding *H9* with other Hessian fly resistance genes in a single genotype.

Key words: *Triticum aestivum* — gene mapping — gene pyramiding — Hessian fly resistance — marker-assisted selection — microsatellite — sequence characterized amplified region

Hessian fly [*Mayetiola destructor* (Say)] is one of the major insect pests of wheat (*Triticum aestivum* L.) worldwide. Genes in wheat that confer resistance to the Hessian fly provide the most efficient and economical means of crop protection against this damaging insect. To date, 32 Hessian fly resistance genes have been identified in wheat and its wild relatives, and these resistance genes have been designated in a series from *H1* to *H32* (Delibes et al. 1997, Ratcliffe and Hatchett 1997, Martín-Sánchez et al. 2003, Williams et al. 2003; and for *H32*, V. Sardesai and C. E. Williams, personal communication). Gene *H6* was located on chromosome 5A by monosomic analysis (Gallun and Patterson 1977). Genes *H3* and *H9* were shown by segregation analysis to be linked to *H6*, and *H15* was shown to be closely linked or allelic to *H9*, forming the linkage block *H3-H6-H9-H15* (Patterson and Gallun 1977, Stebbins et al. 1982, Maas et al. 1989). Gene *H9* confers resistance against Hessian fly biotype L, the most virulent and prevalent biotype in eastern USA.

Gould (1986) predicted that the resistance of a cultivar containing multiple genes for resistance to a single biotype of the Hessian fly could be effective up to 20 times longer than resistance of a cultivar with a single resistance gene. However, the phenotypes of plants containing pyramided genes conferring resistance to biotype L are phenotypically indistinguishable as expression of one gene masks the presence of others. Pyramiding of resistance genes can be efficiently achieved only by employing molecular markers that co-segregate with the respective resistance genes (Williams et al. 2003). An RAPD marker linked to *H9* in wheat was identified using wheat near-isogenic lines (NILs) (Dweikat et al. 1997). However, marker-assisted selection (MAS) with RAPD markers is not always feasible. In addition, some RAPD markers lack reliability. To address these limitations associated with the RAPD marker $OPO05_{1000}$ research was initiated to convert the RAPD polymorphic fragment that co-segregated with gene *H9* to a sequence characterized amplified region (SCAR), in which the longer primers designed from the polymorphic fragment resulted in an allele-specific marker.

To date, few Hessian fly resistance genes have been mapped or characterized by molecular markers (Williams et al. 2003). The lack of linked and mapped molecular markers has limited the utility of these genes for germplasm enhancement and cultivar development by MAS. Thus, the objectives of this study were to: (i) convert an RAPD marker linked to the Hessian fly resistance gene *H9* into a SCAR marker and (ii) map *H9* in the wheat genome to facilitate future MAS in wheat for Hessian fly resistance.

Materials and Methods

Plant materials: The plant materials used for this study consisted of *T. aestivum* L. cultivars 'Newton' and 'Len', and a series of wheat cultivars/lines differing for the presence of several Hessian fly resistance genes (Table 1). 'Newton' and 'Len' are susceptible to all known biotypes of the Hessian fly. Wheat lines 'Ella' (Patterson et al. 1982) and 'Iris' (Patterson et al. 1994) both have Hessian fly resistance gene *H9*. An F_2 population (population 1) of 118 individuals was developed from a cross of 'Ella' × 'Len'. A second population (population 2) of 135 individuals was derived from a cross of 'Iris' × 'Len'.

Wheat line Chinese Spring (CS) and 21 CS nulli-tetrasomic (NT) lines (Sears 1966), where nullisomy for a specific chromosome is

Table 1: Fragment size of markers in 16 wheat cultivars and lines with or without known Hessian fly resistance genes after amplification with the markers linked to *H9*

Cultivar/line	Resistance gene	SOPO05$_{909}$	Fragment length (bp)		
			Xcfa2153	*Xgwm136*	*Xbarc263*
'Len'		−	225	340	240
'Newton'		−	208	Null	240
'Erin'	*H5*	−	215	Null	200
'Caldwell'	*H6*	−	205	360	240
'Ella'	*H9*	+	175	280	220
'Iris'	*H9*	+	175	280	220
'Joy'	*H10*	−	220	Null	200
Nwt207	*H11*	−	190	Null	200
'Luso'	*H12*	−	225	Null	200
'Molly'	*H13*	−	210	Null	200
8395A1	*H14*	−	208	370	240
IN81602C50	*H15*	−	220	350	220
921682A4	*H16*	−	190	Null	240
921680D1	*H17*	−	202	335	200
'Parker76'	*H18*	−	228	345	200
PI422297	*H19*	−	235	360	200

−, absent; +, present.

compensated for by two extra copies of a homoeologue, and deletion lines 1AS-1 (KSU#4510-1) and 1AS-3 (KSU#4510-3) of CS were also included in this study.

DNA isolation: Genomic DNA was isolated from seedling leaves using the CTAB method described by Saghai-Maroof et al. (1984) with minor modifications. A 1.67% CTAB extraction buffer [100 mM Tris-HCl buffer pH 8.0, 1.67% (w/v) hexadecyltrimethylammonium bromide (CTAB), 100 mM Na_2EDTA, and 1.4 M NaCl] was used. DNA concentration was quantified on a Hoefer DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech Inc., Dubuque, IA, USA).

Screening for resistance: F_2 populations together with parents (controls) were seeded in soil in wooden flats. Seedlings were infested with biotype L 1 week after emergence. Three weeks after infestation, F_2 plants and parents were classified as resistant or susceptible. Hessian fly resistance was evaluated as described by Ohm et al. (1995).

Cloning and sequencing of the target RAPD DNA fragment: Polymerase chain reaction (PCR) was carried out with DNA of the wheat line 'Ella', primer OPO05 and with the reaction conditions as described by Dweikat et al. (1997). The gel slice containing the target RAPD fragment was excised from the agarose gel using a sharp-edged clean razor blade, and then eluted with 30 µl of 1x TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0). From this, 1 µl aliquots of the selected excised RAPD products were re-amplified under the same conditions. The amplified products were separated by electrophoresis at 70 V in a 1.2% low-melting point agarose gel. The critical fragment was excised from the gel and purified using QIAquick gel-extraction Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. An aliquot of 3.0 µl of the purified DNA was ligated into a pGEM®-T easy vector (Promega, Madison, WI, USA) according to the procedures described by the manufacturer with minor modifications. The host strain DH5α was used as competent cells for transformation. The recombinant plasmids were plated on selective media LB containing ampicillin and X-gal.

QIAprep® Miniprep Kit (Qiagen Inc.) was used for plasmid DNA extraction. PCR amplification was performed using 25 µl total volume of 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM each of T7 and SP6 primers, 1 unit of *Taq* polymerase, 10 ng of template DNA to check for the presence of the target insert. The amplification profile consisted of one cycle at 94°C for 2 min, followed by 36 cycles of 45 s at 94°C, 1 min at 62°C and 1.5 min at 72°C, with a final extension of 7 min at 72°C.

All 10 samples selected from a single transformation plate showed a single amplification product in a 1.2% agarose gel immersed in 0.5x TBE buffer (90 mM Tris-Borate, 1 mM EDTA, pH 8.0). The 10 corresponding purified plasmid DNAs were sent to the DNA Sequencing Laboratory at the Genomic Center (Purdue University) for sequencing. Similarity searches were performed using the BLAST algorithm at http://www.ncbi.nlm.nih.gov of the National Center for Biotechnology Information (NCBI), with the program BLASTN.

SCAR primer design and allele-specific PCR amplification: The following oligonucleotide primers, designed from the identical sequence of plasmid DNA, led to polymorphisms between the parents and the resistant and susceptible bulks: primer-forward: 5′-CCCAGTCACTC-ATATGCTACCTAT-3′ and primer-reverse: 5′-CCGAGTTGATAT-GCACGATG-3′.

The 5′-end of the forward primer contained all 10 bases of RAPD primer OPO05 (5′-CCCAGTCACT-3′), but the reverse primer was designed differently from the RAPD primer to avoid possible secondary structure or primer dimer generation and false priming (Fig. 1). The optimal PCR amplification was conducted using 25 µl reactions containing 40 ng of template DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.8 mM $MgCl_2$, 200 µM of each dNTP, 0.3 µM of each primer and 1 U of *Taq* DNA polymerase. After an initial heat denaturation step at 94°C for 2 min, DNA fragment amplification was performed for 35 cycles comprising 45 s at 94°C, 1 min at 56°C and 1.5 min at 72°C. Final extension was for 7 min at 72°C. To separate the amplified products, 2.0% agarose gels stained with ethidium bromide were used and the products were visualized by illumination with ultraviolet light.

Chromosomal location of *H9*: The specific DNA fragment amplified from 'Ella' by the SCAR marker was used as probe for hybridization to a Southern blot of CS and CS nulli-tetrasomic lines to determine its chromosome location. For this analysis, 20 µg of genomic DNA of CS and its nulli-tetrasomic lines digested with *Hin*dIII were separated in a 0.8% agarose gel in 1.0x TBE buffer and blotted onto Hybond N^+ nylon membrane by an alkaline procedure (0.4 N NaOH). The probe was labelled by a random prime labelling system using the *redi*Prime™ II kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The membrane was pre-hybridized at 65°C in 6× SSPE, 5× Denhardt's solution, 0.5% (w/v) SDS and 50 µg/ml denatured salmon sperm DNA for 6 h, and hybridized with probe (5 ng/ml) in 6× SSPE, 5× Denhardt's, 0.5% (w/v) SDS, 0.05 µg/ml denatured salmon sperm DNA and 0.05 µg/ml Dextran sulfate overnight at 65°C. The membrane was washed at 65°C once in 2× SSPE containing 0.5% (w/v) SDS for 30 min, once in 1× SSPE containing 0.5% (w/v) SDS for 30 min, and once in 0.5× SSPE containing 0.5% (w/v) SDS for 30 min.

Bulked segregant analysis: For bulked segregant analysis (BSA) (Michelmore et al. 1991), equivalent amounts of genomic DNA from 15 resistant and 15 susceptible F_2 plants from the population derived from the cross 'Ella' × 'Len' were respectively pooled to form resistant and susceptible bulks. Both bulks were used along with the parents to identify markers showing polymorphisms between the four samples. These polymorphic markers were further used to analyse individual F_2 plants to determine linkages between SSR/STS markers and the resistance gene *H9*.

Microsatellite and STS analysis: Wheat microsatellite markers designated as either *Xgwm* for Gatersleben (Germany) wheat microsatellite (Röder et al. 1998) or *Xgdm* for Gatersleben D-genome microsatellite (Pestsova et al. 2000), selected to cover chromosome 1A based on the Southern analysis, were tested for useful polymorphisms in parents and bulks. When the approximate chromosomal arm location of gene *H9* was confirmed by amplifying genomic DNA of 1AS deletion lines (KSU#4510-1 and KSU#4510-3) with the specific SSR markers and the specific primers (forward primer 5′-GAAGACAAACGGTGG-GAGAA-3′ and reverse primer 5′-CGGCGTACATAGTCGTTCC-3′)

CCCAGTCACTCATATGCTACCTATATATGTTGAAGTATGTAATCATGTCGA
GTCATTCTAGGTTTTATTTCTAGCAAAAATAAGAACCGGTCTATGCAGCGT
GAGCGTGTTCATGACACGGCCGGATCAATAGCTGCATGTTGTCACGATCG
GCTCATGGGATGGCACGAGTACTGGCCGTAGCGGCTTAGAACTTGAGGCA
TGATCAAGGGAAGTAGGATGGCACGTCCATGAAGCTTGATCAAGGGAAG
TAGGATGGCACGTCCATGAAGCTTGATCATGGTGCACGAATAGGATCTTT
ATGTAGTTAATTGAGCTAATGAGCTATTTAAATAAAGAGGAGAACAAAGC
AAAGCAGAAAAGTTGCAACGTTTTACACGACACAAATATCCTGAGTGTTC
ATTGATTCTTCTCCCTCCCCTCTCTGCTCCAATAATATATACCTCTGGATGA
GTTCATATGTCTCATCCT***GAAGACAAACGGTGGGAGAA***GGCACCTGCGGG
GGTGAGGTGTGCTACTCTGGTCTTTGCTTTCGCAGCGGTTGCAGGTGCCAG
TACCCCTACTGCTGGAAGCGCCCAACGCCTCCTGCTTGAAGCCCTCCCATG
CATGCATCAACCCTAAAATGTT***GGAACGACTATGTACGCCG***TGGTCGCGTG
CTTTCTTTCTCAAGCTACCACTATACACGAATGTGAACTATGGTATGTGTG
GCTCTCGTGCCTGCAGTCAGGAGATGTGGATAAGAGCGTGGTGGTGTTGT
AATACCTATGTTGGTGGTTAAATAAATGGCATGCGTGTTGTATGTGTTATG
TCTCGTGTAACTCAAATGTATACATTTGTACTATGTTCATGTGGTTATAAA
TGTCTGCTTGCCGCAAGTTTTGCATCTTTCATCGTGCATATCAACTCGGGT
TCCTCCTACATGCATATACGAATTGATTATTCCGTCAAAATACTAAAGAAC
GTATATTCCTGTTGAGTGATGAGATAGTGACTGGG

Fig. 1: The DNA sequence of the RAPD fragment from $OPO05_{1000}$. The regions used for the SCAR primers are underlined, and the RAPD primers for $OPO05_{1000}$ are shaded. The specific primers designed for the 1AS deletion line test are in bold italic

designed from the $OPO05_{1000}$ (Fig. 1), additional markers including *Xbarc* (Beltsville Agriculture Research Center), *Xksum* (Kansas State University microsatellite), *Xcnl* (Cornell University microsatellite), *Xcfa* and *Xcfd* (Pierre Sourdille microsatellite), and *Xpsp* (John Innes Research Centre microsatellite) on chromosome 1A also were tested on the *H9* population from the cross 'Ella' × 'Len'. Considering that both *Pm3* and *Lr10* are located on chromosome 1AS, the STS marker for *Pm3* (*STS-Pm3*, forward primer 5′-ATGGCTAGATGCCCGTTATG-3′ and reverse primer 5′-AGAGCAGAGCAGTGCAACAA-3′) and the STS marker for *Lr10* (*STS-Lr10*, forward primer 5′-GCGCTATGCCTAACCTGAAG-3′ and reverse primer 5′-CTCCACATAGGCAGCACTGA-3′) were developed based on the available sequences from the GenBank database (GenBank nos AY605285 and AY270157 respectively). SSR, STS and SCAR primers were designed by Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) and synthesized by Integrated DNA Technologies (Coralville, IA, USA).

PCR for each SSR and STS marker was performed in a PTC-100 Thermal Cycler (MJ Research, Watertown, MA, USA) at amplifications of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 50, 52, 55 or 60°C (based on primer annealing temperature) for 40 s, and 72°C for 1 min, with a final extension at 72°C for 7 min before cooling to 4°C. Each PCR reaction (25 μl) consisted of 40 ng of template DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.25 μM of each primer and 1 U of *Taq* DNA polymerase. The amplified PCR products were fractionated on 2.0–3.0% agarose gels (based on the size difference of the polymorphism) using a mixture of 1:1 Metaphor® and Seakem® in 0.5x TBE buffer and photographed over a UV light source.

Linkage analysis: Data were analysed using the chi-square test to ascertain goodness-of-fit between the expected ratio for a single dominant gene and the observed phenotypic segregation. Linkage analysis between the SSR or other PCR-based markers and the *H9* resistance locus was performed with the software package MAPMAKER/EXP version 3.0 (Lander et al. 1987). Map units were computed by applying the Kosambi function (Kosambi 1944). An LOD score of 3 and the maximum distance of 50 cM were used in the determination of linkages.

Results

Hessian fly response phenotyping

F_2 populations 1 ('Ella' × 'Len') and 2 ('Iris' × 'Len') segregated, respectively, 89 resistant : 29 susceptible and 105 resistant : 30 susceptible. These numbers fit a 3 : 1 ratio ($\chi^2 = 0.01$, $P > 0.90$ and $\chi^2 = 0.35$, $P > 0.50$ respectively) of a single, dominant gene for Hessian fly resistance in both 'Ella' and 'Iris'. The tests to phenotype the two F_2 populations were definitive; all plants of the resistant parents, 'Ella' (*H9H9*) and 'Iris' (*H9H9*), were clearly not stunted and all plants of the susceptible parent, 'Len', were clearly stunted. Also, the F_2 populations segregated in the expected ratio of 3 resistant : 1 susceptible.

Conversion of RAPD marker $OPO05_{1000}$ into a SCAR marker

The RAPD OPO05 critical DNA fragment was amplified from the DNA of 'Ella' (*H9H9*) but not from the Hessian fly-susceptible line 'Newton' (data not shown) and the expected 997-bp critical fragment was sequenced (Fig. 1). Of the 10 colonies from a single transformation plate, all the resulting

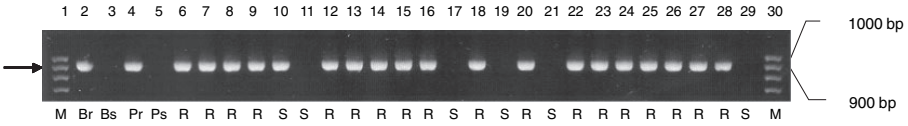

Fig. 2: DNA bands amplified from parents, bulks and 24 F_2 plants derived from a cross between the resistant wheat cultivar 'Ella' (*H9H9*) and 'Len' (*h9h9*) with SCAR marker $SOPO05_{909}$ shown in a 2.0% agarose gel. M, Pr, Ps, Br, Bs, R and S represent the 100-bp DNA ladder, resistant parent, susceptible parent, resistant bulk, susceptible bulk, and resistant and susceptible individual F_2 plants respectively. The 909-bp DNA fragment amplified from the resistant parent 'Ella', resistant bulk, resistant F_2 plants, and the putative recombinant F_2 plant in lane 10, is indicated by the arrow on the left

sequences contained the $OPO05_{1000}$ primer at both 5′ and 3′-ends. Seven inserts had an identical 997-bp sequence following a multiple alignment produced by the software Clustal X (Jeanmougin et al. 1998) (Fig. 1). Oligonucleotide primers were designed based on that sequence in an effort to develop a site-specific or SCAR marker. These primers identified a 909-bp band in the resistant parent 'Ella', but no amplicon in the susceptible line 'Newton'.

The same primers were used to screen the parents, resistant and susceptible bulks, and individual F_2 plants of population 1. The expected 909-bp DNA fragment was amplified from the resistant parent, resistant bulk and resistant individual F_2 plants, whereas no DNA fragment was amplified from the susceptible parent, bulk and F_2 plants, except the putative recombinant plants (Fig. 2). Linkage analysis performed using Mapmaker indicated that this SCAR marker (hereafter referred to as $SOPO05_{909}$) was linked with the resistance gene *H9* (Fig. 3). Similar results were obtained with F_2 population 2. The 909 bp amplification product was present in all resistant individuals, but was not present in susceptible plants, except the putative recombinant plants.

SCAR primers were used to amplify DNA from cultivars/lines containing other Hessian fly resistance genes located on chromosomes 1A and 5A. A 909-bp fragment was detected only in the resistant wheat lines, 'Ella' and 'Iris', containing *H9* (Table 1). Thus, the RAPD marker was converted into a dominant SCAR marker designated as $SOPO05_{909}$ meaning SCAR marker of size 909 bp derived from RAPD primer OPO05.

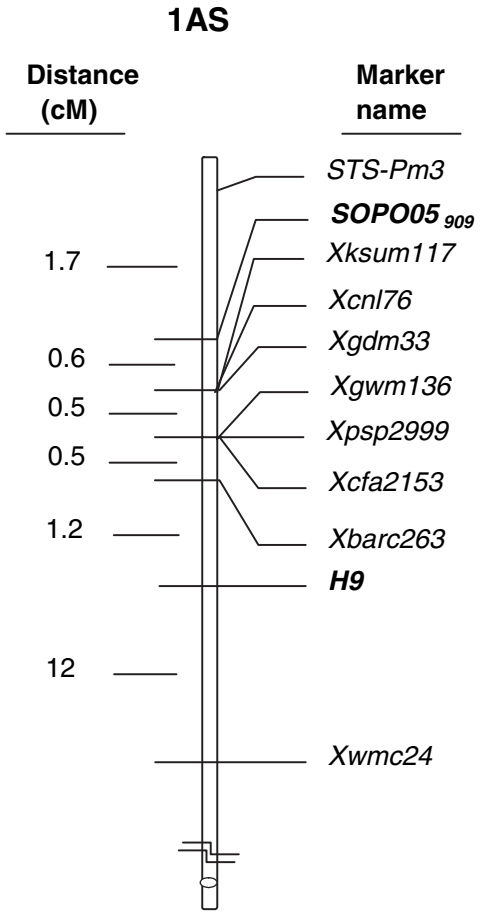

Fig. 3: Linkage map of the short arm of wheat chromosome 1A showing the genetic location of the Hessian fly resistance gene *H9*. Markers were mapped in 118 F_2 individuals from a cross between the resistant cultivar 'Ella' (*H9H9*) and the susceptible 'Len' (*h9h9*)). Approximate distances in centimorgans (cM) and molecular markers are indicated on the left and the right, respectively. The letter *X* in front of each SSR locus name indicates the basic symbol for a molecular marker with unknown function

Chromosomal location of *H9*

The 'Ella'-specific SCAR fragment ($SOPO05_{909}$) was eluted from the gel and purified using QIAquick gel-extraction Kit. Southern hybridization of *Hin*dIII-digested genomic DNAs of CS and CS nulli-tetrasomic lines with the $SOPO05_{909}$ fragment as probe revealed that one band about 1.5 kb was missing in N1A-T1D. In contrast, the other nulli-tetrasomic lines had the same banding patterns as CS (data not shown). This indicated that $OPO05_{909}$ is located on chromosome 1A.

Molecular mapping of *H9*

To test the hypothesis that $SOPO05_{909}$ and *H9* are on chromosome 1A, population 1 was further genotyped with several SSR markers that have been mapped to this region of chromosome 1A and that were determined to be polymorphic between the parent lines 'Ella' and 'Len'. All these loci showed 1 : 2 : 1 segregation in the F_2 population (Table 2), indicating linkage between *H9* and other marker loci on chromosome 1A. Recombination analysis indicated that the Hessian fly resistance gene *H9* was flanked by *Xbarc263* and *Xwmc24* with map distances of 1.2 and 12 cM respectively. Nine loci were located distally to *H9* on chromosome 1AS. No recombination was observed among *Xgwm136* (Fig. 4), *Xpsp2999* and *Xcfa2153* or among *Xksum117*, *Xcnl76* and *Xgdm33* in this population, so the first group, *Xgwm136*, *Xpsp2999* and *Xcfa2153*, mapped to the same position on the short arm of chromosome 1A, and was 1.7 cM distal to gene *H9*. The second group of markers, *Xksum117-1A*, *Xcnl76-1A* and *Xgdm33-1A*, mapped to another position 2.2 cM distal to the *H9* gene (Fig. 3). These two groups were only 0.5 cM apart. $SOPO05_{909}$ and *STS-Pm3* were also linked to *H9*, with linkage

Table 2: Segregation analysis for the *H9* locus and molecular markers in an F_2 population from the cross 'Ella' × 'Len'

Gene or markers	No. of F_2 plants	Observed no.			Expected ratio	χ^2	P
		H9H9[1]	*H9h9*[1]	*h9h9*[1]			
H9 (phenotype)	118	89[2]		29	3 : 1	0.01	> 0.90
$SOPO05_{909}$	118	90[2]		28	3 : 1	0.10	0.75–0.90
Xgwm136	118	37	53	28	1 : 2 : 1	2.59	0.10–0.20
Xgdm33	118	38	53	27	1 : 2 : 1	3.27	0.05–0.10
Xbarc263	118	34	55	29	1 : 2 : 1	0.97	0.25–0.50
Xpsp2999	118	37	53	28	1 : 2 : 1	2.59	0.10–0.25
STS-Pm3	118	38	50	30	1 : 2 : 1	3.83	0.05–0.10
Xwmc24	118	32	61	25	1 : 2 : 1	0.97	0.25–0.50
Xksum117	118	38	53	27	1 : 2 : 1	3.27	0.05–0.10
Xcnl76	118	38	53	27	1 : 2 : 1	3.27	0.05–0.10
Xcfa2153	118	36	54	28	1 : 2 : 1	1.55	0.20–0.25
Xgwm33	118	35	83[3]		1 : 3	1.37	0.20–0.25
Xcfd15	118	35	83[3]		1 : 3	1.37	0.20–0.25
Xcnl137	118	37	81[3]		1 : 3	2.54	0.10–0.25

[1]Genotype: *H9H9* = 'Ella'; *H9h9* = heterozygous; *h9h9* = 'Len'.
[2]Pooled values from resistant homozygous and heterozygous classes.
[3]Pooled values from heterozygous and susceptible homozygous classes.

Fig. 4: DNA bands amplified from parents, bulks and 24 F_2 plants derived from a cross between the resistant cultivar 'Ella' (*H9H9*) and 'Len' (*h9h9*) with microsatellite primer pair *Xgwm136* shown in a 3.0% agarose gel. M, Pr, Ps, Br and Bs represent the 20-bp DNA ladder, resistant parent, susceptible parent, resistant bulk and susceptible bulk respectively. Resistant homozygous, heterozygous, and susceptible homozygous are indicated by R, H and S respectively. The 275 bp DNA fragment amplified from the resistant parent 'Ella', resistant bulk and resistant F_2 plants is indicated by the arrow on the left

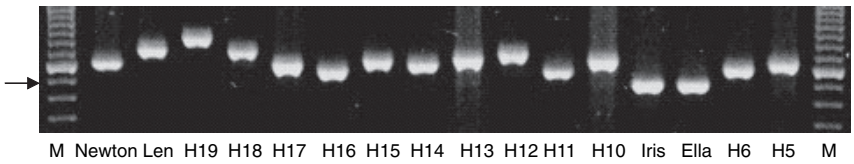

Fig. 5: DNA bands amplified from cultivars/lines with or without known Hessian fly resistance genes (Table 2) with microsatellite primer pair *Xcfa2153* shown in a 3.0% agarose gel. M presents a 20-bp DNA ladder used as a standard size marker. The 175 bp DNA fragments amplified from 'Ella' and 'Iris', both *H9H9*, are indicated by the arrow on the left

distances of 2.8 and 4.5 cM respectively (Fig. 3). Unfortunately, *STS-Lr10* did not show polymorphism in the BSA analysis. Surprisingly, three more distal SSR markers, *Xgwm33-1A*, *Xcfd15-1A* and *Xcnl137-1A*, showed polymorphism between resistant 'Ella' and susceptible 'Len' (the distinguishing band in the gel is present in the susceptible parent 'Len', and is not present in the resistant parent 'Ella'), but they do not distinguish (they are dominant markers) susceptible homozygous plants from heterozygous plants in the F_2 population (Table 2), fitting a 1 : 3 segregation ratio ($\chi^2 = 1.37$, $0.20 < P < 0.25$; $\chi^2 = 1.37$, $0.20 < P < 0.25$; $\chi^2 = 2.54$, $0.10 < P < 0.25$; respectively), and as a result could not be mapped with respect to the *H9* resistance locus. Because of their co-dominant inheritance and close linkage to *H9*, the microsatellite markers, *Xbarc263-1A* and *Xgwm136-1A*, were chosen to detect their location in relation to *H9* using CS deletion lines of 1AS (KSU#4510-1 and KSU#4510-3). Analysis of genomic DNA of 1AS deletion lines with the SSR primers for loci *Xbarc263* and *Xgwm136* along with the specific primers from $OPO05_{1000}$ confirmed that all the loci tested and, presumably, the closely linked *H9* gene were located on chromosome 1AS (data not shown).

To validate the linked markers for MAS, DNA of 14 resistant cultivars/lines with known Hessian fly resistance genes and two susceptible cultivars were amplified using the microsatellite markers *Xcfa2153*, *Xgwm136* and *Xbarc263* (Table 1). The same 220-bp allele at *Xbarc263* as that in 'Ella' and 'Iris' was present in wheat line IN81602, which has Hessian fly resistance gene *H15*, shown by Maas et al. (1989) to be closely linked or allelic to *H9*. The amplified fragments in all other genotypes carrying known Hessian fly resistance genes were different from the amplified fragment in 'Ella' and 'Iris'. This indicated that the 220-bp allele at *Xbarc263* was not specific to gene *H9*. In contrast, the critical fragments with sizes of 175 and 280 bp amplified by *Xcfa2153* (Fig. 5) and *Xgwm136*, respectively, were only amplified in 'Ella' and 'Iris', containing *H9* (Table 1), indicating that the marker loci *Xcfa2153-1A* and *Xgwm136-1A* are linked to gene *H9*.

Discussion

'Ella' carries the dominant Hessian fly resistance gene *H9*, which was initially localized on chromosome 5A by segregation analysis, showing linkage to *H6* (Stebbins et al. 1982). *H6* was localized on chromosome 5A by segregation analysis using wheat monosomics (Gallun and Patterson 1977). A SCAR marker, $SOPO05_{909}$, that is closely linked to *H9*, was developed here. Southern hybridization of *Hin*dIII-digested genomic DNAs of CS and CS nulli-tetrasomic lines with the $SOPO05_{909}$ fragment as probe revealed that $OPO05_{909}$ and, presumably, the closely linked *H9* were located on chromosome 1A. Further evidence of *H9* being located on chromosome 1AS came from the results of PCR amplification obtained from two CS deletion lines of 1AS (KSU#4510-1 and KSU#4510-3) with the primers from $OPO05_{1000}$ and the closely linked SSR markers *Xgwm136* and *Xbarc263*.

Bulked segregant analysis with markers on 1AS identified *Xbarc263* and *Xwmc24* as flanking the *H9* locus at distances of 1.2 and 12 cM respectively. In the present study, marker loci *Xgwm136*, *Xpsp2999* and *Xcfa2153* co-segregated at approximately 1.7 cM distal to *H9*; the marker loci *Xgdm33*, *Xcnl76* and *Xksum117* co-segregated at a location approximately 2.2 cM distal to *H9*. However, the linkage relationships among these markers have been somewhat different in previous studies. For example, *Xgdm33*, *Xcfa2153* and *Xpsp2999* co-segregated in an RI population derived from a cross between the wheat cultivars 'Arina' and 'Forno' (Paillard et al. 2003). In another study, gene *Pm3g* was shown to co-segregate with *Xpsp2999*, and was 2.3 cM distal to *Xgdm33* (Bougot et al. 2002). Discrepancies in marker locations among these studies are probably due to different parent lines, differences in population type and size, and discrepancies in phenotyping.

There are various reports that resistance genes to different pests and pathogens are linked and located in clusters observed in wheat (McIntosh et al. 1995, 2003, Adhikari et al. 2004), rice (Sardesai et al. 2002), maize (Hulbert et al. 2001), tomato (Dickinson et al. 1993) and soybean (Ashfield et al. 1998, Bachman et al. 2001). The genomic region that contains *H9* is also particularly rich in genes for resistance against fungal pathogens. For example, *Pm3* for resistance to wheat powdery mildew (incited by *Blumeria graminis*) was also mapped with RFLP marker *BCD1434* (Hartl et al. 1993, Ma et al. 1994) and SSR marker *Xpsp2999* (Bougot et al. 2002) to the distal region of the short arm of chromosome 1A. RFLP marker *BCD1434* was tightly linked to *Pm3*. At least 10 alleles (*Pm3a–Pm3j*) were identified at this locus (Zeller and Hsam 1998). The present study also confirmed that the STS marker derived from the powdery mildew resistance gene *Pm3* was linked to the Hessian fly resistance gene *H9* at a genetic distance of 4.5 cM. The leaf rust resistance gene *Lr10*, effective against *Puccinia triticina* Eriks, was also mapped in the same chromosomal region as *H9* (Schachermayr et al. 1997, Guyot et al. 2004). Unfortunately, the *STS-Lr10* marker did not show polymorphism between the parent lines in the present study. However, because most of these markers on 1AS showed linkage with *Pm3*, *Lr10* and *H9*, it is possible to deduce a likely arrangement of these genes in relation to the molecular markers in the distal region of chromosome 1AS. It appears that a likely order of the resistance genes may be: the telomere – *Pm3* – *H9* – *Lr10* (Hartl et al. 1993, Ma et al. 1994, Schachermayr et al. 1997, Bougot et al. 2002, Paillard et al. 2003). The presence of multiple disease resistance genes and the positional cloning for both *Lr10* and *Pm3b* in bread wheat (Stein et al. 2000, Feuillet et al. 2003, Yahiaoui et al. 2004) make this genomic region much more attractive for future analyses and even possible map-based cloning of the Hessian fly resistance gene *H9*.

Efficient pyramiding of effective Hessian fly resistance genes is only possible with the aid of markers linked to the resistance genes. To achieve this goal, a simple but efficient method is needed to identify different Hessian fly resistance genes in a broad genetic background typical of, and necessary for, successful breeding programmes. The results of this study are of practical significance to Hessian fly resistance breeding. The specific and diagnostic SSR markers closely linked to *H9* identified in this study not only can assist wheat breeders in making parental selection but will also facilitate pyramiding the Hessian fly resistance genes into elite breeding lines during cultivar development. These markers, plus others already mapped (Dweikat et al. 1997, 2002, Seo et al. 1997, Williams et al. 2003), will speed the construction of breeding lines containing different resistance loci to develop broad-spectrum and durable resistance to Hessian fly. Although the *Xbarc263-1A* locus was tightly linked to the *H9* locus and could be useful in transferring this gene into improved wheat cultivars by MAS, identification of additional molecular markers with tighter linkages and which flank the *H9* gene would be desirable to improve selection efficiency, particularly considering the lower level of polymorphism on the proximal side of *H9*.

The error in placing gene *H6* on chromosome 5A, rather than on 1A by monosomic analysis could be the result of any of a number of factors including chromosome shift or misidentification of monosomic genetic stocks. However, mapping of *H6*, originally and erroneously on chromosome 5A, emphasizes the importance of correct initial mapping of a gene when it is used subsequently as a reference point for mapping additional genes. Genes *H3* (Patterson and Gallun 1977) and *H9* (Stebbins et al. 1982) were located by their linkage to *H6*, and *H15* was shown to be closely linked or allelic to *H9* (Maas et al. 1989). The original location of *H6* was never validated and all subsequent locations to the same region were based on close linkage to *H6*. The DNA marker analysis here places *H9* on the short arm of chromosome 1A. Further work is required to verify if all members of the previously reported linkage block *H3-H6-H9-H15* (Patterson and Gallun 1977, Stebbins et al. 1982, Maas et al. 1989) are located on the short arm of chromosome 1A.

Acknowledgements

The authors acknowledge the financial support from USDA-IFAFS competitive grant 2001-04462, USDA-ARS and Purdue University. They are also grateful to Dr B. S. Gill, Kansas State University, for providing the seeds of wheat nulli-tetrasomic and deletion lines of 1AS.

References

Adhikari, T. B., H. Wallwork, and S. B. Goodwin, 2004: Microsatellite markers linked to the *Stb2* and *Stb3* genes for resistance to *Septoria tritici* blotch in wheat. Crop Sci. **44,** 1403—1411.

Ashfield, T., J. R. Danzer, D. Held, K. Clayton, P. Keim, M. A. Saghai Maroof, D. M. Webb, and R. W. Innes, 1998: *Rpg1*, a soybean gene effective against races of bacterial blight, maps to a cluster of previously identified disease resistance genes. Theor. Appl. Genet. **96,** 1013—1021.

Bachman, M. S., J. P. Tamulonis, C. D. Nickell, and A. F. Bent, 2001: Molecular markers linked to brown stem rot resistance genes, Rbs_1 and Rbs_2, in soybean. Crop Sci. **41,** 527—535.

Bougot, Y., J. Lemonine, M. T. Pavoine, D. Barloy, and G. Doussinault, 2002: Identification of a microsatellite marker associated with *Pm3* resistance alleles to powdery mildew in wheat. Plant Breeding **121,** 325—329.

Delibes, A., F. J. Delmoral, J. A. Martinsanchez, A. Meijias, M. Gallego, D. Casado, E. Sin, and I. Lopezbrana, 1997: Hessian fly resistance gene transferred from chromosome 4M (V) of *Aegilops ventricosa* to *Triticum aestivum*. Theor. Appl. Genet. **94,** 858—864.

Dickinson, M. J., D. A. Jones, and J. D. G. Jones, 1993: Close linkage between *CF2/Cf5* and *mi* resistance loci in tomato. Mol. Plant Microbe Interact. **6,** 341—347.

Dweikat, I., H. Ohm, F. Patterson, and S. Cambron, 1997: Identification of RAPD markers for 11 Hessian fly resistance genes in wheat. Theor. Appl. Genet. **94,** 419—423.

Dweikat, I., W. Zhang, and H. Ohm, 2002: Development of STS markers linked to Hessian fly resistance gene *H6* in wheat. Theor. Appl. Genet. **105,** 766—770.

Feuillet, C., S. Treavella, N. Stein, L. Albar, A. Nublat, and B. Keller, 2003: Map-based isolation of the leaf rust disease resistance gene *Lr10* from the hexaploid wheat (*Triticum aestivum* L.) genome. Proc. Natl Acad. Sci. USA **100,** 15253—15258.

Gallun, R. L., and F. L. Patterson, 1977: Monosomic analysis of wheat for resistance to Hessian fly. J. Hered. **68,** 223—226.

Gould, F., 1986: Simulation models for predicting durability of insect-resistant germ plasm: Hessian fly (Diptera: Cecidomyiidae)-resistant winter wheat. Environ. Entomol. **15,** 11—23.

Guyot, R., N. Yahiaoui, C. Feuillet, and B. Keller, 2004: In silico comparative analysis reveals a mosaic conservation of genes within a novel colinear region in wheat chromosome 1AS and rice chromosome 5S. Funct. Integr. Genomics **4,** 47—58.

Hartl, L., H. Weiss, F. J. Zeller, and A. Jahoor, 1993: Use of RFLP markers for the identification of alleles of the *Pm3* locus conferring powdery mildew resistance in wheat (*Triticum aestivum* L.). Theor. Appl. Genet. **86,** 959—963.

Hulbert, S. H., C. A. Webb, S. M. Smith, and Q. Sun, 2001: Resistance gene complexes: evolution and utilization. Annu. Rev. Phytopathol. **39,** 285—312.

Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins, and T. J. Gibson, 1998: Multiple sequence alignment with Clustal X. Trends Biochem. Sci. **23,** 403—405.

Kosambi, D. D., 1944: The estimation of map distances from recombination values. Ann. Eug. **12,** 172—175.

Lander, E. S., P. Green, J. Abrahamson, A. Barlow, M. J. Daly, S. E. Lincoln, and L. Newburg, 1987: Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics **1,** 174—181.

Ma, Z. Q., M. E. Sorrells, and S. D. Tanksley, 1994: RFLP markers linked to powdery mildew resistance genes *Pm1*, *Pm2*, *Pm3*, and *Pm4* in wheat. Genome **37,** 871—875.

Maas, F. B., F. L. Patterson, J. E. Foster, and H. W. Ohm, 1989: Expression and inheritance of resistance of ELS6404-160 durum wheat to Hessian fly. Crop Sci. **29,** 23—28.

Martín-Sánchez, J. A., M. Gómez-Colmenarejo, J. Del Moral, E. Sin, M. J. Montes, C. González-Belinchón, I. López-Braña, and A. Delibes, 2003: A new Hessian fly resistance gene (*H30*) transferred from the wild grass *Aegilops triuncialis* to hexaploid wheat. Theor. Appl. Genet. **106,** 1248—1255.

McIntosh, R. A., C. R. Wellings, and R. F. Park, 1995: Wheat Rusts: An Atlas of Resistance Genes. CSIRO Publications, East Melbourne, Australia.

McIntosh, R. A., Y. Yamazaki, K. M. Devos, J. Dubcovsky, W. J. Rogers, and R. Appels, 2003: Catalogue of gene symbols for wheat. In: N. E. Pogna, M. Romano, E. A. Pogna, and G. Galterio (eds), Proc. 10th Int. Wheat Genetics Symp., Instituto Sperimentale per la Cerealcoltura, Rome, Vol. 4, On CD.

Michelmore, R. W., I. Paran, and R. V. Kesseli, 1991: Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl Acad. Sci. USA **88,** 9828—9832.

Ohm, H. W., H. C. Sharma, F. L. Patterson, R. H. Ratcliffe, and M. Obanni, 1995: Linkage relationships among genes on wheat chromosome 5A that condition resistance to Hessian fly. Crop Sci. **35,** 1603—1607.

Paillard, S., T. Schnurbusch, M. Winzeler, M. Messmer, P. Sourdille, O. Abderhalden, B. Keller, and G. Schachermayr, 2003: An integrative genetic linkage map of winter wheat (*Triticum aestivum* L.). Theor. Appl. Genet. **107,** 1235—1242.

Patterson, F. L., and R. L. Gallun, 1977: Linkage in wheat of the *H3* and *H9* genetic factors for resistance to Hessian fly. J. Hered. **68,** 293—296.

Patterson, F. L., R. L. Gallun, N. B. Stebbins, and S. K. Cambron, 1982: Registration of Stella and Ella common wheat germplasm lines. Crop Sci. **22,** 902—903.

Patterson, F. L., F. B. Mass III, J. E. Foster, R. H. Ratcliffe, S. Cambron, G. Safranski, P. L. Taylor, and H. W. Ohm, 1994: Registration of eight Hessian fly –resistant common winter wheat germplasm lines (Carol, Erin, Flynn, Iris, Joy, Karen, Lola and Molly). Crop Sci. **34,** 315—316.

Pestsova, E., M. W. Ganal, and M. S. Röder, 2000: Isolation and mapping of microsatellite markers specific for the D genome of bread wheat. Genome **43,** 689—697.

Ratcliffe, R. H., and J. H. Hatchett, 1997: Biology and genetics of the Hessian fly and resistance in wheat. In: K. Bondari (ed.), New Developments in Entomology: Research Signpost, 47—56. Scientific Information Guild, Trivandrum, India.

Röder, M. S., V. Korzun, K. Wendehake, J. Plaschke, M. H. Tixier, P. Leroy, and M. W. Ganal, 1998: A microsatellite map of wheat. Genetics **149,** 2007—2023.

Saghai-Maroof, M. A., K. M. Soliman, R. A. Jorgensen, and R. W. Allard, 1984: Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance chromosomal location and population dynamics. Proc. Natl Acad. Sci. USA **81,** 8014—8018.

Sardesai, N., A. Kumar, K. R. Rajyashri, and S. Nair, 2002: Identification and mapping of an AFLP marker linked to *Gm7*, a gall midge resistance gene and its conversion to a SCAR marker for its utility in marker aided selection in rice. Theor. Appl. Genet. **105,** 691—698.

Schachermayr, G., C. Feuillet, and B. Keller, 1997: Molecular markers for the detection of the wheat leaf rust resistance gene *Lr10* in diverse genetic backgrounds. Mol. Breed. **3,** 65—74.

Sears, E. R., 1966: Nullisomic-tetrasomic combinations in hexaploid wheat. In: R. Riley, and K. R. Lewis (eds), Chromosome Manipulation and Plant Genetics, 29—45. Oliver and Boyd, Edinburgh.

Seo, Y. W., J. W. Johnson, and R. L. Jarret, 1997: A molecular marker associated with the *H21* Hessian fly resistance gene in wheat. Mol. Breed. **3,** 177—181.

Stebbins, N. B., F. L. Patterson, and R. L. Gallun, 1982: Interrelationships among genes *H3*, *H6*, *H9*, and *H10* for Hessian fly resistance. Crop Sci. **22,** 1029—1032.

Stein, N., C. Feuillet, T. Wicker, E. Schlagenhauf, and B. Keller, 2000: Subgenome chromosome walking in wheat: a 450-kb physical contig in *Triticum monococcum* L. spans the *Lr10* resistance locus in hexaploid wheat (*Triticum aestivum* L.). Proc. Natl Acad. Sci. USA **97,** 13436—13441.

Williams, C. E., C. C. Collier, N. Sardesai, H. W. Ohm, and S. E. Cambron, 2003: Phenotypic assessment and mapped markers for *H31*, a new wheat gene conferring resistance to Hessian fly (Diptera: Cecidomyiidae). Theor. Appl. Genet. **107,** 1516—1523.

Yahiaoui, N., P. Srichumpa, R. Dudler, and B. Keller, 2004: Genome analysis at different ploidy levels allows cloning of the powdery mildew resistance gene *Pm3b* from hexaploid wheat. Plant J. **37,** 528—538.

Zeller, F. J., and S. L. K. Hsam, 1998: Progress in breeding for resistance to powdery mildew in common wheat (*Triticum aestivum* L.). In: A. E. Slinkard (ed.), Proc. 9th. Int. Wheat Genet. Symp., Vol. 1, 178—180. Saskatoon, Saskatchewan, Canada.